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REVIEW

Causes of decreased photosynthetic rate and metabolic capacity in water-deficient leaf cells: a critical evaluation of mechanisms and integration of processes

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• *Background* Water deficit (WD) decreases photosynthetic rate (A) via decreased stomatal conductance to CO_2 (g_s) and photosynthetic metabolic potential (A_{pot}). The relative importance of g_s and A_{pot}, and how they are affected by WD, are reviewed with respect to light intensity and to experimental approaches.
• *Scope and Conclusions* With progressive WD, A decreases as g_s falls. Under low light during growth and WD, A is stimulated by elevated CO_2, showing that metabolism (A_{pot}) is not impaired, but at high light A is not stimulated, showing inhibition. At a given intercellular CO_2 concentration (C_i) A decreases, showing impaired metabolism (A_{pot}). The C_i and probably chloroplast CO_2 concentration (C_c), decreases and then increases, together with the equilibrium CO_2 concentration, with greater WD. Estimation of C_c and internal (mesophyll) conductance (g_i) is considered uncertain. Photosystem activity is unaffected until very severe WD, maintaining electron (e^-) transport (ET) and reductant content. Low A, together with photorespiration (PR), which is maintained or decreased, provides a smaller sink for e^-, causing over-energization of energy transduction. Despite increased non-photochemical quenching (NPQ), excess energy and e^- result in generation of reactive oxygen species (ROS). Evidence is considered that ROS damages ATP synthase so that ATP content decreases progressively with WD. Decreased ATP limits RuBP production by the Calvin cycle and thus A_{pot}. Rubisco activity is unlikely to determine A_{pot}. Sucrose synthesis is limited by lack of substrate and impaired enzyme regulation. With WD, PR decreases relative to light respiration (R_L), and mitochondria consume reductant and synthesise ATP. With progressing WD at low A, R_L increases C_i and C_c. This review emphasises the effects of light intensity, considers techniques, and develops a qualitative model of photosynthetic metabolism under WD that explains many observations: testable hypotheses are suggested.

Key words: Water stress, photosynthesis, photorespiration, stomata, ATP synthase, ATP, photoinhibition, electron transport, Rubisco, fluorescence, sucrose, mesophyll conductance.

INTRODUCTION

This review aims to advance understanding of the effects of relatively rapidly developing water deficit (WD) in plants on photosynthetic rate (A). It complements reviews by Chaves and Oliviera (2004) and Chaves *et al.* (2009), which provide a wider perspective. Considerable effort (see Boyer, 1990; Kramer and Boyer, 1995; Lawlor and Cornic, 2002) has already been devoted to analysing the effects of WD on A via stomatal conductance (g_s) and photosynthetic potential (A_{pot}) in different experimental systems, but there is lack of consensus over their relative importance. For example, Tezara *et al.* (1999) and Medrano *et al.* (2002) have concluded that g_s and metabolism (RuBP and ATP supply) limit A at low WD, whereas Flexas *et al.* (2004*a*) and Chaves and Oliviera (2004) have concluded that g_s decreases A and that metabolic limitations are unimportant, or only at severe WD.

Reductionist science might seem to require a single factor determining the effects of WD on photosynthetic rate and processes; however, finding such an elusive *deus ex machina* in very complex, partial and imperfect data requires some faith (or dogmatism). Identifying 'a cause' of decreased A, and A_{pot}, under WD under all environmental conditions may not be possible because the concept is incorrect. Photosynthetic systems are varied, structurally and functionally dynamic, and dependent on the environment, which is also extremely dynamic (Lawlor, 2001). Control of A and A_{pot} is distributed between many metabolic components and processes that vary in importance as conditions – environmental and within the plant – change (von Caemmerer, 2000). Probably control varies, depending on the plant and on environmental conditions during growth and under water deficit. Hence different experiments produce different answers. Understanding effects of WD on photosynthesis will come from better quantification of the interactions between WD (and other environmental conditions) and photosynthetic mechanisms.

Photosynthesis and water deficit: why the emphasis?

Photosynthetic CO_2 assimilation per unit area and time, i.e. rate (A), is inhibited by rapidly developing WD in physiological studies, so it is assumed to be responsible for decreased dry matter production. However, leaf area is also very important for total production. This applies in the field, where slowly developing WD results in a small leaf area index, which often dominates

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production with only small effects on A (Legg *et al.*, 1979; Sinclair and Purcell, 2005). However, the perceived need to apply understanding of photosynthesis to alleviation of practical problems such as loss of crop yield due to WD has increased interest in 'water stress physiology'. Many basic questions remain about how cellular processes are regulated by WD. As A dominates cell metabolism, with very large fluxes of carbon, nitrogen and energy (Lawlor, 2001), it is potentially vulnerable to WD (Kramer and Boyer, 1995). It is integrated with respiration and aspects of electron transport (ET) and ATP synthesis in the mitochondria (Atkin and Macherel, 2009), and changes in A and energy are related, in ways still unclear, to accumulation of 'stress metabolites' (e.g. proline), gene expression and protein synthesis. It is now appreciated (Herbert, 2002; Scheibe *et al.*, 2005; Rumeau *et al.*, 2007) how tightly integrated photosynthetic metabolism is, and how difficult it will be, without understanding of the system and a clear model, to engineer plants for large biomass and yield production under WD (Sinclair and Purcell, 2005; Bohnert *et al.*, 2006). It is also extremely doubtful if one model of photosynthesis and metabolism will suit all plant × environment combinations (Reynolds *et al.*, 2005). Differences between plants grown in controlled environments and those in the field must be considered, especially when considering the potential for genetic modification (Sinclair and Purcell, 2005). Quantitative assessment of conditions in photosynthetic cells under WD is essential if the current flood of information from genomics, proteomics and metabolomics is to be used to improve plant production under WD (Flexas *et al.*, 2004*b*; Chaves *et al.*, 2009). First, general agreement on the qualitative processes involved is required, from which quantitative species × environment models may emerge.

Is there a standard experimental system?

Before discussing WD and its effects on A and A_{pot}, it is essential to assess the conditions of experiments. Standardization is minimal: studies are very disparate in terms of species, environment and mode of applying stress and hence comparison of data is difficult (Table 1). Few studies have been done in variable field conditions (e.g. Legg *et al.*, 1979; Wise *et al.*, 1991, 1992; Escalona *et al.*, 1999; Tezara *et al.*, 2003; Ripley *et al.*, 2007), some with plants grown in pots (Quick *et al.*, 1992). Controlled environments are not necessarily qualitatively and quantitatively more uniform for plants grown in small pots. Disparities between experiments and selective emphasis on particular techniques (Table 1) generate very different data from which conflicting conclusions have been drawn, fuelling much competition between concepts (Lawlor, 2002; Flexas *et al.*, 2004*a*).

Experimental conditions. From Table 1 (which focuses on C_3 plants such as sunflower, wheat and bean) we identify four general types of experimental approaches.

(1) Plants are grown under glasshouse or controlled-environment conditions, often at low light, and samples of leaf are taken and subjected to WD under no or low light, resulting in rapid stress (Kaiser and Heber, 1981; Dietz and Heber, 1983; Kaiser 1984, 1987; Renou *et al.*, 1990; Tourneux and Peltier, 1995).
(2) Plants are grown as above, but subjected to WD under particular conditions before sampling for experimentation: generally with stronger light (Tang *et al.*, 2002).
(3) Plants are grown hydroponically as in (1) with relatively defined water status (Ψ, RWC) applied rapidly (over hours to days) using osmotica (e.g. polyethylene glycol); measurements are made on intact plants, light may differ between studies (Lawlor and Fock, 1975; Lawlor, 1976; Renou *et al.*, 1990; Zhou *et al.*, 2007).
(4) Plants dry a small volume of soil, resulting in progressive decrease in Ψ, RWC, osmotic potential and turgor over several days, during which measurements are made. The rates of decrease in Ψ and RWC depend, amongst other things, on environment, leaf area and g_s. However, they do not change linearly with duration or with soil water content because of the soil water characteristic curve (Kramer and Boyer, 1995). This complicates experimentation (Sinclair and Purcell, 2005), requiring well-replicated measurements, sampling, etc, under comparable conditions, generally within a single experiment.

There is no preferred species, although sunflower is frequently used. *Arabidopsis* has been little used despite its importance in molecular biology, because of technical difficulties in measuring gas exchange. Thus no standard or model system has been adopted. The great importance of environmental factors, and their interactions with the plant, is often ignored (or not appreciated). Differences in duration and severity of WD interacting with the intensity and duration of light are particularly important. A standardized approach is urgently required (Blum, 1999) for physiological and molecular studies of WD if the current confusion in the literature is to be remedied.

WATER DEFICIT AND PHOTOSYNTHESIS

The schematic in Fig. 1 emphasizes some of the most important cellular structures, metabolic processes and fluxes that determine photosynthesis and are affected by WD. Boyer and co-workers (see Kramer and Boyer, 1995; Tang *et al.*, 2002) in particular have contributed greatly to analysis of the effects of WD on photosynthesis, and Cornic and Briantais (1991), Lawlor and Cornic (2002), Lawlor (2002), Flexas *et al.* (2004*a*) and Chaves *et al.* (2009), have also considered 'the problem'. Here, we focus on:

(1) metabolic potential for photosynthesis (A_{pot}), which is determined by the capacity of the system related to the amounts and activities of components of light-harvesting, electron transport and energy-transduction processes, and of carbon metabolism, including enzymes (e.g. Rubisco) and processes (RuBP synthesis), of the Calvin cycle.
(2) rate of photosynthesis (A), which is determined by stomatal and internal limitations (g_s and g_i, respectively) to CO_2 diffusion and A_{pot} (Tezara *et al.*, 1999; Lawlor, 2002; Lawlor and Cornic, 2002).

The distinction between A and A_{pot} is not just semantic and is sometimes confused as 'photosynthesis' may refer to both. WD affects both A via changes in g_s (and possibly g_i) and through A_{pot}: the former is related largely to tissue water and

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TABLE 1. *Analysis of experiments from which data have been used to assess the effects of water deficit on photosynthetic metabolism. Particular attention should be paid to the conditions during growth (col. 3) and application of water deficit (col. 4). Note also that the types of measurements (col. 5) are related to the data derived and thus the interpretation, for example of sub-stomatal CO_2 concentration (C_i). Chloroplastic CO_2 concentration (C_c) and the ratio of C_i to atmospheric CO_2 concentration (C_a) are indicated in columns 6–8. Col. 9 assesses the effects of removal of the epidermis and/or elevated CO_2 on photosynthesis. Col. 10 shows the A/C_i response with ↓ indicating decreased slope and plateau. Col. 11 comments on the role of stomata in controlling photosynthesis under water deficit. Col. 12 indicates incorporation of alternative sinks into calculation of C_c by estimating electron flux. Col. 13 contains a general evaluation of experimental data*

				Measurements taken?									
Reference	Species	Growth conditions / PAR (μmol m^{-2} s^{-1}) / Duration (h d^{-1})	Water deficit conditions / PAR / Duration	CO_2 / O_2 / Fluorescence	C_i	C_c	C_i/C_a	CO_2 / Epidermis removal stimulates A?	A/C_i	g_s controls A?	Metabolism inhibited?	PR / Alt. sinks?	Comments
Lawlor & Fock, 1975	*Helianthus*	CE / 400 / 16	PEG / hours	Y / N / N						Y, mild WD	Y, moderate/ severe WD		PR decreasing, R_D increasing
Kaiser 1981, 1984	*Spinacia*	Unkown	Osmotic / 200 / mins	Y / Y / N Leaf slices	N	N	–	Y / Y		Y, to severe WD	Y, severe WD Photophos-phorylation?		Conditions artificial Primary reactions insensitive. Calvin cycle decreased severe WD
Dietz & Heber, 1983	*Primula palinari*	Greenhouse / low ??? or 200? / ?	Leaf pieces / 0 or 200 / fast	Y / N / N Leaf slices	N	N	–	Y / Y		Y, to severe WD	Y, at severe WD		Low light, rapid WD, effect on CO_2 assimilation not light reactions or R_D
Sharp & Boyer, 1986	*Helianthus*	CE / 900 /14	Soil / 4 d 40 or 2000 / 6 hr	Y / N / N Attached leaf	Y		Decrease/ increase		↓	Y, mild but not severe WD	Y, moderate WD		Metabolic inhibition. No photoinhibition
Cornic *et al.*, 1987	*Phaseolus*	CE / 310 / 16	Soil / 4–8 d	Y / N / Y Attached leaf	Y	N	Decrease?/ increase			Y, mild WD	Y, moderate WD		Metabolic inhibition, C_i/C_a rises large WD
Kirschbaum, 1987	*Eucalyptus*	Greenhouse, natural Canberra / high? / 12	Soil	Y / N / N Attached leaf	Y	N	Decrease/ increase	N	↓	Y, mild WD	Y, progressive		Decreased A/C_i, no major PI
Cornic *et al.*, 1989	*Phaseolus*	CE / 200 / 16	Soil / 200? 15 d	Y / Y / Y Attached leaf Y / Y / Y	Y	Y		Y		Y, to severe WD	Y, very severe WD		C_i misleading Random leaves measured over period
	Elatostema	CE / 40 / 16	Soil / 25 d	Attached leaf	Y	Y		Y					
Renou *et al.*, 1990	*Triticum*	CE / 600 / 14	PEG / hours	Y / Y / N	Y? ↓ to comp. pt	Y	?	Y / –		Y	N, moderate WD Y, very severe WD	Y/N	C_i errors? C_c low assumes only PR
Cornic & Briantais, 1991	*Phaseolus*	Greenhouse / 350? / ?	Soil / 8 d	Y / N / Y Attached leaf	Y ↓ to comp. pt	Y ↓ to comp. pt	Constant/ Increase?			Y, to severe WD	N, moderate WD Y, severe WD	Y/Y?	Fluorescence calculation gives low C_c but not measured
Giménez *et al.*, 1992	*Helianthus*	Greenhouse + CE / 450 / 16	Soil / 450 / 4 d	Y / N / N Attached leaf	Y	N	Decrease/ increase	N/-	↓	Y, mild WD	Y, moderate WD		RuBP limiting A/C_i

Continued

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TABLE 1. *Continued*

Reference	Species	Growth conditions / PAR (μmol m^{-2} s^{-1}) / Duration (h d^{-1})	Water deficit conditions / PAR / Duration	Measurements taken?										
				CO_2 / O_2 / Fluorescence	C_i	C_c	C_i/C_a	CO_2 / Epidermis removal stimulates A?	A/C_i	g_s controls A?	Metabolism inhibited?	PR / Alt. sinks?	Comments
Martin & Ruiz-Torres, 1992	*Triticum*	CE / 500 / 16	Soil / 500 / ?days	Y / Y / N Attached leaf	Y	N	–		↓	Y, mild WD	Y, moderate WD		Metabolic limit Calvin cycle
Lauer & Boyer, 1992	*Helianthus Glycine, Phaseolus*	CE / 600–800 / 14	Soil / 12 d	Y / N / N Attached leaf	Y	N	Small decrease/ then increase	N/-		Y, mild WD	Y, mild WD		Direct measure C_i, decrease/increase
Quick *et al.*, 1992	*Lupinus*, *Helianthus*, *Vitus*, *Eucalyptus*	Field Portugal, natural / high?	Soil pots / up to 6 d	Y / N / N	Y	N	Large decrease	Y/-		Y, mild to moderate WD	N, mild to moderate WD		Stomatal control only *Lupin*, *Helianthus*, *Eucalyptus*. Metabolic *Vitus*, large sucrose
Tourneux & Peltier, 1995	*Solanum*	CE / 600 / 12 Much water	Discs /dark 0 / hours ?	N / Y / Y Discs	N	Y ↓ to comp. pt	Y			Y	N, or very low WD	Y/N	Extreme conditions assumes PR only
Escalona *et al.*, 1999	*Vitus*	Field / large/ 14	Drought vs.irrigated / days	Y / N / N Attached leaf	Y	N	Decrease		↓	Y, mild WD	Y, moderate/ severe WD		RuBP limiting A/C_i
Tezara *et al.*, 1999, 2008	*Helianthus*	Greenhouse–CE / 500–700/ 14	Soil / 400 / 8 d	Y / Y / Y Attached leaf	Y	N	Decrease/ increase	N / –	↓	Y, mild WD	Y, moderate/ severe WD	Y/N	ATP limiting RuBP and A/C_i
Wingler *et al.*, 1999	*Hordeum*	CE / 460 / 12	Soil / 12 d	Y / N / Y	Y	N	–			??	Y, moderate WD		C_i measured not given. PR indirect evidence not increased
Tang *et al.*, 2002	*Helianthus*	Glasshouse, large / ? / 14 CE / 700–900/ 12	Soil / several days	N / Y / N Leaf pieces	N	N	–	N / N		Y, mild WD	Y, mild WD		Metabolic limit
Haupt-Herting & Fock, 2002	*Lycopersicum*	CE / 200 / 16	Soil/ 200 / 8 d	Y / Y / Y Attached leaf	Y	N	–	N / –		Y, mild WD	Y, moderate WD	Y/Y	PR increased relative but not absolute sink for e^- with WD

Botha *et al.*, 2004	*Rhamnus, Nicotiana, Vitus*	Greenhouse / 600 / 14?	Soil / 600 / variable	Y / N / Y Attached leaf	Y	N	Variable		↓ Y, mild WD	Y? severe WD, Rubisco limit	Combined data, large errors
Ennahli & Earl, 2005	*Gossypium*	Glasshouse / winter / 10	Soil / ? / several days	Y / N / Y Attached leaf	Y	Y ↓ to comp. pt	Decrease/ increase	N	↓ Y, mild WD	Y, progressive	Y/N	Calculation of C_c based on fluorescence assuming only PR. Conflicts in data
Flexas *et al.*, 2006	*Glycine*	CE / 800–1000 / 14	Soil /?	Y / N / Y Attached leaf	N	Y ↓ to comp. pt	–		Y, to severe WD	Y, severe WD, Rubisco	Y/N	Calc C_c based on fluorescence assuming only PR
	Nicotiana			Attached leaf								
Zhou *et al.*, 2007	*Oryza*	CE / 400 / 500 / 12	PEG / 500 / 2d	Y / N / N Attached leaf	Y	N	Decrease/ increase	Y mild	↓ Y, mild WD	Y, mild WD		ROS increase mild stress
Ripley *et al.*, 2007	*Alloteropsis*	Field–polytunnel / large / 400+ / 12	Soil / 400 / several days	Y / N / Y Attached leaf	Y	Y	Decreased (mild WD)	N / –	↓ Y, mild WD	Y, mild/ moderate WD,		Limited WD, high light, RuBP/Rubisco limiting

the latter to metabolism under the conditions prevailing in the chloroplast and cell. Discrepancies in the literature concerning regulation of A revolve around the relative effects of WD on g_s and g_i and on A_{pot}, and what causes them. Considering C_3 mesophytes, in Fig. 2 we have summarized and simplified information from the literature (see Table 1) in order to obtain an overview of changes in amounts, fluxes, etc, of some key components in relation to RWC: this emphasizes general cell water relations and does not assume a mechanism. Recently, emphasis has been placed on g_s (Medrano *et al.*, 2002; Flexas *et al.*, 2004*a*) because it is regarded as the controlling factor. This over-emphasizes g_s and under-estimates cellular water status and metabolic factors, which are driving forces determining A and A_{pot}. By its very nature, g_s is highly variable, being affected by physiological state (e.g. leaf water status) and environment (e.g. water vapour pressure, CO_2 concentration) and so is not a satisfactory basis for comparison. It is as if electrical circuits were analysed in terms of variable resistances, ignoring electrical potential (voltage). In addition, g_s reaches a minimum below which much important cell activity occurs, so presenting data on this basis distorts the metabolic responses. Because of the (incompletely) known importance of cellular conditions, evaluation of all limiting factors (of both A and A_{pot}) and cell metabolism is required, preferably by appropriate experimental and statistical techniques.

When water loss from leaves exceeds uptake, WD develops. With a small decrease in RWC of approx. 10–20 %, turgor (P) decreases from 0·7–0·9 to 0 MPa, and Ψ from 0 to −1 MPa (Fig. 2A). Concomitantly, g_s (Fig. 2B) and, as a consequence of limitation to CO_2 diffusion, A (Fig. 2C) decrease substantially (approx. 30–50 %). This much is generally agreed (Cornic *et al.*, 1987; Cornic and Briantais, 1991; Cornic *et al.*, 1992; Lawlor 1995, 2002; Tezara *et al.*, 1999; Flexas *et al.*, 2004*a*). However, the decrease in metabolism shown by A_{pot} (Fig. 2G; see section on A/C_i curves, below) that occurs (Tezara *et al*, 1999; Lawlor 2002) is not observed or accepted by, for example, Cornic and Briantais (1991), Quick *et al.* (1992), Cornic (2000), Cornic and Fresneau (2002) and Flexas *et al.* (2004*a*), who consider that g_s determines A, even at large WD. The view is that A_{pot} is sustained, so that with small g_s and g_i, C_i and C_c decrease substantially, approaching or reaching the compensation point and are responsible for decreasing A (Cornic and Fresneau, 2002). Before discussing this, the role of stomata is considered.

Stomatal conductance under water deficit

Changes in g_s depend on hydraulic factors (RWC, Ψ and turgor) in the stomatal apparatus, including transport of water across membranes (which involves aquaporins; Kaldenhoff *et al.*, 2008), and metabolic (e.g. ABA-related) processes (Comstock, 2002; Buckley, 2005; Roelfsema and Hedrich, 2005). Changes in g_s may be rapid, occurring within minutes of alterations in the atmospheric humidity or alterations in the root-medium water- or osmotic potential. This serves to regulate water loss in relation to uptake, so RWC decreases very little. In experimental system (4) listed above, an initial approx. 50 % decrease in g_s is related to a decrease of approx. 10 % in RWC and 0·5 MPa in Ψ

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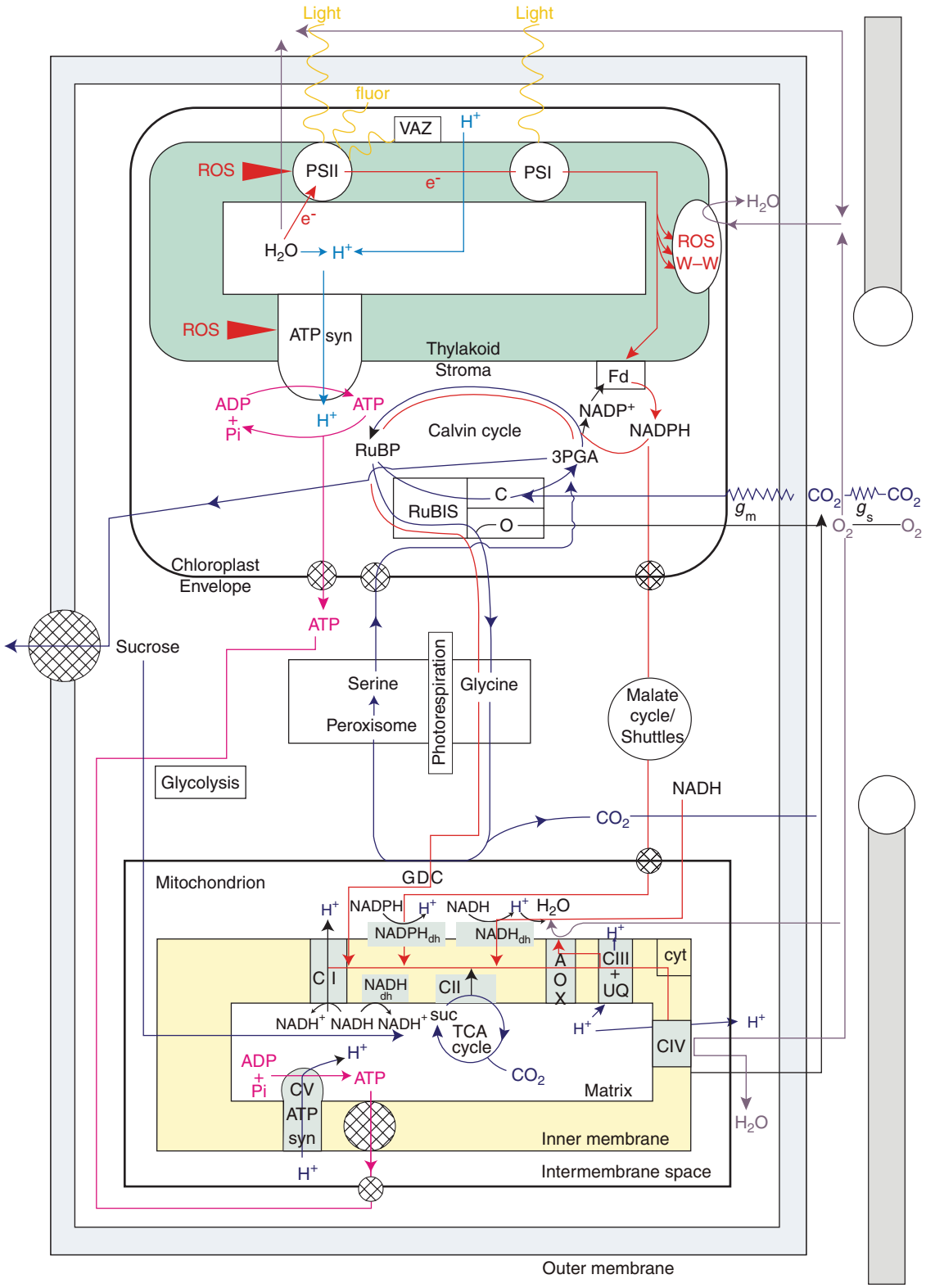

FIG. 1. Photosynthesis in a C_3 leaf under water deficit involves all of the main structures in the compartments of the mesophyll cell. This greatly simplified schematic attempts to provide an overview (see Lawlor, 2001) of the structures and the associated energy, electron, carbon and oxygen fluxes. Light (yellow) is captured by chlorophyll in the antennae and photosystems (PS), and the energy excites the reaction centres. Excited PSII passes electrons (e^-, red arrows) to PSI via a chain of redox components, reducing them. Simultaneously, e^- is removed from water and passes to PSII and O_2 is released (purple arrows). H^+ accumulates in the thylakoid lumen (together with H^+ transferred from the cytosol into the lumen by ET; light blue arrows). Passage of H^+ through ATP synthase generates ATP from ADP and P_i. ET reduces ferredoxin (Fd) and then $NADP^+$, forming NADPH: ATP and NADPH are used by the Calvin cycle to generate RuBP (dark blue), which reacts with CO_2 from the atmosphere, catalysed by the enzyme Rubisco (carboxylase reaction shown

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(Fig. 2A). Effects may be rapidly reversible, or longer-term and persistent. ABA, possibly at small concentrations either transported from roots or released from 'storage' in the leaf, may interact with hydraulic regulation initially. Most likely, ABA is synthesised *de novo* and accumulates substantially as turgor is lost (approx. 80 % RWC and −1 MPa decrease in Ψ; Pierce and Raschke, 1980; Cornish and Zeevaart, 1984) and may then dominate regulation, ensuring long-term closure. Regulation of g_s is integrated with photosynthetic metabolism and the environment in ways not well understood (Buckley, 2005). A g_s substantially smaller than the unstressed value is probably not a long-term solution to WD under field conditions (Legg *et al.*, 1979), unless there is rapid and major adjustments in all aspects of the mechanisms for dealing with excess energy (see Noctor *et al.*, 2002) and imbalance between supply and demand of assimilates and organ growth. Expansion of cells and tissues is rapidly slowed by a small WD and stops at zero P (80–90 % RWC). This must alter the balance between photosynthetic assimilate supply and demand. Under field conditions, with slowly developing WD, plants often do not exhibit large decrease in g_s. Rather, long-term adjustment involves smaller leaf area index (LAI). For example, a barley crop growing in the field as WD developed slowly (over many weeks) avoided low RWC by decreasing LAI and thereby water loss, and by changing cell water balance rather than closing stomata. However, a much larger crop under rapid drought responded with decreased RWC and metabolic inhibition (Legg *et al.*, 1979). The composition and function of the photosynthetic system changes during development, and depends ultimately on control of gene expression (Pfannschmidt *et al.*, 2009), allowing some adaptation to conditions, including WD.

Effects of elevated C_a *on* A *and* A/C_i *curves*

Evaluation of the effects of WD on A_{pot} has come from assessing the response of A to increasing external CO_2 concentration (C_a) and calculated C_i. The view that g_s determines A, even at substantial WD (70–60 % RWC or less), rests on studies where removing the epidermis (and thus g_s) or increasing C_a overcomes the limiting g_s (Kaiser and Heber, 1981; Dietz and Heber, 1983; Kaiser 1984, 1987; Quick *et al.*, 1992; Tourneux and Peltier, 1995; Cornic, 2000). However, a pattern is apparent (see Table 1, and references therein): these studies used plants grown at low irradiance with WD that developed quickly in no or weak light before measurement. In contrast, similar experiments but using plants grown at higher irradiance (Table 1) show that removal of the epidermis and increasing C_a (Tang *et al.*, 2002) do not restore A to unstressed values, showing that A_{pot} is impaired – a result considered by Flexas *et al.* (2004*a*) to be caused by low RWC. Similarly, application of elevated C_a (up to such large concentrations that metabolism was inhibited) failed to reverse the decrease in A (Tezara *et al.*, 1999). Haupt-Herting and Fock (2002) were unable to reverse decreased A of intact and attached leaves with a ten-fold increase in C_a. Zhou *et al.* (2007) reversed inhibition with increased C_a under mild WD but not severe. These results show that WD under low light had most effect via g_s with little or no effect on A_{pot}. In contrast, with stronger light A is not restored by elevated C_a, showing inhibition of A_{pot}. An exception (Quick *et al.*, 1992) is the maintenance of O_2 production with very large (15 %) C_a in *Eucalyptus*, lupin and sunflower, but not in *Vitis*, possibly because of very active stomatal control. It is likely that the effects of g_s and A_{pot} depend on conditions, e.g. the rate and severity of the WD (which also depends on species) and the radiation, with A_{pot} increasing in importance as WD increases under physiologically more relevant conditions (Lawlor, 2002).

Extension of such analyses by measurement of A/C_i responses (curves) on intact plants has provided valuable information. Many studies with relatively long periods of strong irradiance during growth and during slow WD in C_3 plants (see Table 1, and Wise *et al.*, 1991; Martin and Ruiz-Torres, 1992; Tezara *et al.*, 1999; Ripley *et al.*, 2007) have observed decreased slopes and plateaux of A/C_i curves, showing inhibition of A_{pot} (Fig. 2G) even at very mild WD (10–15 % loss of RWC; Table 1). Cornic *et al.* (1987) also observed this, but did not consider that A_{pot} was inhibited. Similarly, Ennahli and Earl (2005) demonstrated a progressive decrease in A/C_i curves with increasing WD; however, the evidence was rejected in favour of substantially decreasing C_c based on fluorescence data, although this may be erroneous [see section on intercellular (mesophyll) conductance, below]. There is further evidence of decreasing A_{pot} from A/C_i curves in the C_3 and C_4 sub-species of *Allopteris semialata*, a grass of southern Africa, even with mild WD (Ripley *et al.*, 2007). Rapidly stressed rice responded similarly (Zhou *et al.*, 2007), showing metabolic limitation.

However, many reasons have been given for not accepting such a large body of consistent, reproducible evidence based on measurements using well-stirred air in leaf chambers on

as RuBIS–C). From the products of this reaction, the carbon flux (dark blue lines) is to sucrose, some of which may be used in darkness for glycolysis and cell respiration, but most is transported (transporter cross-hatched) to the rest of the plant. Rubisco also catalyses the reaction of RuBP with O_2 (oxygenation, shown as RuBIS–O), which ultimately gives rise in the peroxisomes to glycine. This is decarboxylated in the mitochondria, the CO_2 produced is photorespiration and e^- is transferred to the mitochondrial ET chain, where ATP is generated, before reducing O_2. In addition, the tricarboxylic acid (TCA) cycle produces CO_2 from substrates ultimately derived from sucrose. It may operate in darkness (dark respiration) or in the light (day respiration) depending on the activity of photosynthesis. Electrons from the TCA cycle enter the mitochondrial ET chain, passing to O_2 and forming water and transporting H^+, which is coupled to ATP synthesis. ATP is used for reactions in the cytosol or chloroplast. In addition reductant (red arrows) from the cytosol (and from the chloroplasts transferred by metabolite – particularly malate – shuttles) may also be oxidized in the mitochondria by NADH and NADPH dehydrogenases, with ET coupled to ATP synthesis. Under water deficit, the stomata close (and internal conductance g_m, termed g_i in the text, may change) thus limiting the flux of CO_2 to the Calvin cycle, leading to shortage of e^- acceptors and slowing ET, even if PR increases as a proportion of CO_2 assimilation and consumes relatively more e^-. Excess excitation in the antennae and PSII leads, via the thylakoid H^+ concentration, to activation of violaxanthin de-epoxidase, which converts excitation energy to heat by non-photochemical quenching (NPQ). Excess energy leads to reduction of O_2 and formation of reactive oxygen species (ROS, large red arrows), which are partly detoxified (ROS W–W) but may accumulate sufficiently to damage components, e.g proteins of PSII and ATP synthase. This impairs ATP synthesis, decreasing RuBP production and hence A_{pot}. Also, low ATP slows protein synthesis and decreases the cell's ability to repair damage caused by ROS, and affects regulation of ion transport. This schematic should be considered together with Fig. 2, which shows the changes in some components and fluxes of CO_2, energy, etc.

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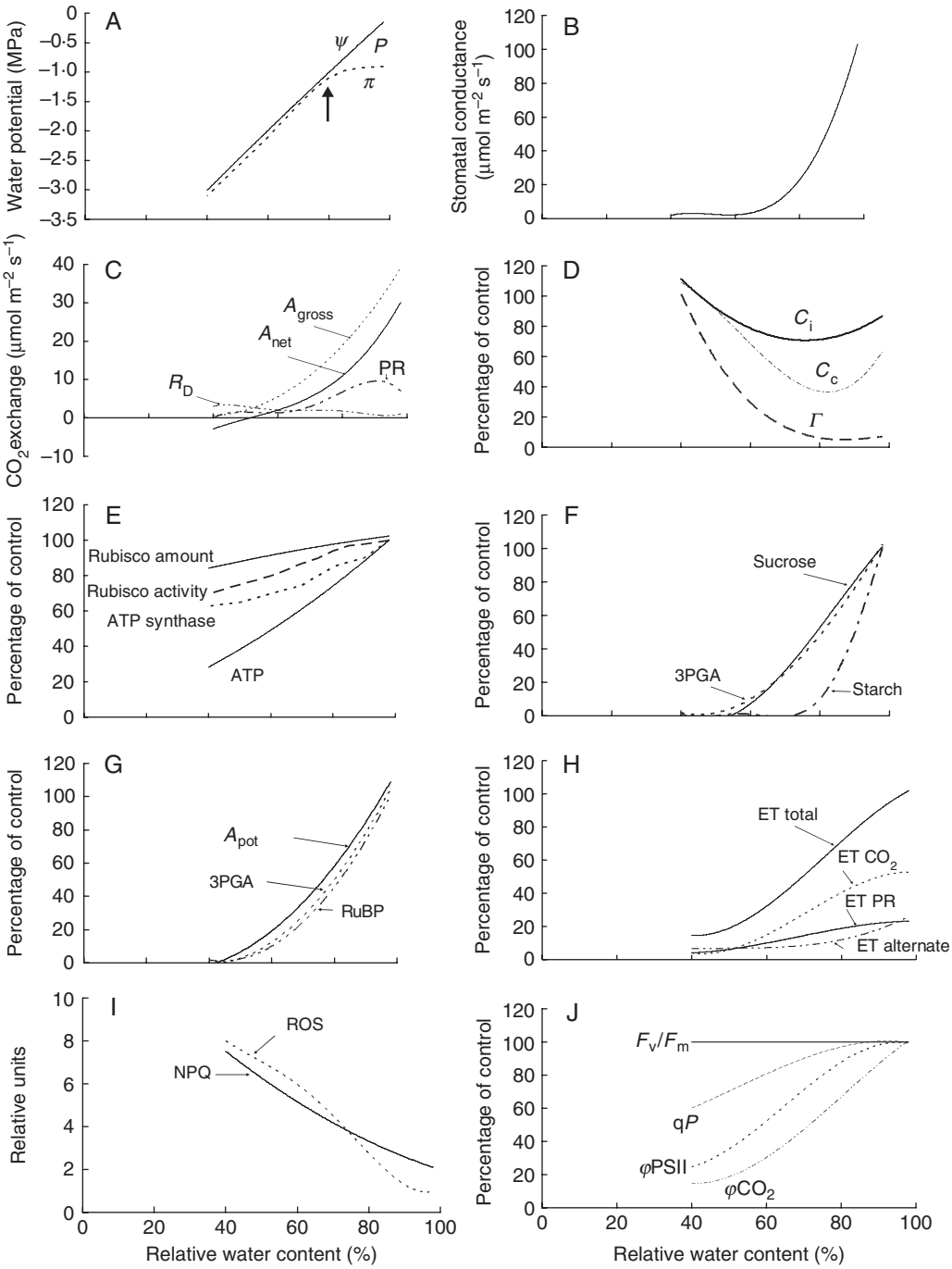

FIG. 2. Representation of measured (or calculated) changes in processes (shown in Fig. 1) of leaves of C_3 plants resulting from water deficit. As the basis of comparison, the relative water content (RWC) is used, as it indicates the sensitivity of processes to changing water status. Information is generalized from the literature (cited in Table 1), for intact (attached) leaves of plants relatively rapidly stressed, under moderate-to-strong light. Relative changes in the components were related to RWC (where necessary derived from ψ, etc., converted assuming published relationships), and averages used to produce the generalized responses shown. The aim is to indicate how processes change with WD: they should be treated as at best semi-quantitative. Accurate relationships of such types are required if analysis of the effects of WD on photosynthesis is to advance. (A) Leaf water potential, ψ, osmotic potential, π, and turgor pressure, P. Zero P is indicated by arrow. (B) Stomatal conductance, g_s. (C) gross and net photosynthetic rate, A, photorespiration PR, and dark respiration, R_D. (D). Concentration of CO_2: substomatal (C_i) and chloroplastic (C_c), and the equilibrium CO_2 compensation concentration (Γ). (E) Total Rubisco protein amount per unit leaf area, and initial Rubisco activity, ATP synthase amount and ATP content of whole leaf. (F) Content of sucrose, 3PGA and starch. (G) Content of RuBP, 3PGA (for comparison) and A_{pot} derived from the plateau of A/C_i curves. (H) Total electron transport and its partitioning to Rubisco carboxylation and oxygenation, and to other sinks. (I) Change in NPQ (measured) and ROS (speculative). (J) Variable to maximal fluorescence (F_v/F_m; efficiency of energy capture of open PSII reaction centres) of dark-adapted leaves, photochemical quenching (qP), quantum efficiency of PSII (ϕPSII) and apparent quantum efficiency of CO_2 assimilation (ϕCO_2).

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well-illuminated, intact leaves droughted when attached to plants. Decreased A_{pot} has been rejected as an artefact of the calculation of C_i (see Cornic and Briantais, 1991; Ennahli and Earl, 2005). This view is supported by reversal of decreased A by elevated C_a on detached (and inevitably damaged) leaf pieces in often unstirred chambers, where water films, large boundary layers and weak light may play a significant role in the responses. Rejection of evidence from attached leaves cites potential errors in measurements of small CO_2 and H_2O fluxes at large WD, although the same methods are accepted for measuring small fluxes, e.g. at low CO_2 and light. Conductance of the cuticle to CO_2 becomes more important as stomata close (Boyer *et al.*, 1997), but has only a small effect even at severe WD (Tezara *et al.*, 1999; Flexas *et al.*, 2004*a*). Other potential technical errors in gas-exchange measurements have been raised (Flexas *et al.*, 2004*a*, 2007; Ennahli and Earl, 2005). Whilst techniques must be questioned, the errors suggested are largely overcome by application of 'best practice' (e.g. tests for leaks, good replication under the same conditions rather than repeats) and are small (Morison *et al.*, 2005, 2007) given the magnitude of the effects of WD.

The most important criticism has come from the perceived role of heterogeneous g_s ('patchy stomata'), which may give erroneous C_i values and thus decrease the slopes and plateaux of the A/C_i curves. Yet theoretical analysis of the distribution of g_s across leaves, and its effect on C_i, is inconclusive (see Buckley *et al.*, 1997). Most importantly, there is no substantial experimental evidence of patchy stomata. Indeed, the evidence suggests (even if proof of a negative is not possible) absence of patchiness (Wise *et al.*, 1991, 1992; Giménez, 1992; Gunasekera and Berkowitz, 1992; Martin and Ruiz-Torres, 1992; Osmond *et al.*, 1999; Haupt-Herting and Fock, 2002). There is continued reluctance to accept this direct evidence (Ennahli and Earl, 2005), although Flexas *et al.* (2004*a*) concede that 'patchiness and cuticular conductance may not totally prevent the usefulness of A/C_i curves'. We conclude that data from A/C_i curves are trustworthy and must be fully considered. They show unequivocally that A_{pot} is impaired by increasing WD in many but not all studies, depending on conditions.

Intercellular and chloroplastic CO_2 concentrations and intercellular (mesophyll) conductance

Consideration of how C_i and C_c change with WD is required as they are central to, and indicate the state of, photosynthetic metabolism.

Intercellular CO_2 concentration. Assimilation of CO_2 by well-watered leaves with large g_s at a C_a of approx. 350 μmol mol^{-1} results in a calculated C_i of aprox. 0·7–0·8 of C_a. As g_s restricts the supply of CO_2, C_i falls to approx. 0·6–0·7 (Fig. 2D), as often observed (e.g. Cornic *et al.*, 1987; Martin and Ruiz-Torres, 1991; Tezara *et al.*, 1999), indicating that A_{pot} is maintained relative to g_s. Hence increasing C_a increases C_i and the plateaux of A/C_i curves, showing stomatal control. However, C_i may not always decrease, e.g. sunflower stressed in the field kept a rather constant C_i as A decreased (Wise *et al.*, 1991). Frequently, following an initial fall, C_i/C_a remains rather constant, and increasing C_i with large C_a does not increase A (Martin and Ruiz-Torres, 1992; Tezara *et al.*, 1999; Haupt-Herting and Fock, 2002), indicating inhibition of A_{pot}. The magnitude of the decrease in C_i differs, presumably because of the relative effects of WD on A, A_{pot}, g_s and respiration, etc. With further loss of RWC (below approx. 70 %) C_i may increase to approach C_a (Cornic *et al.*, 1987) or reach (Ennahli and Earl, 2005) and eventually exceed it (Lawlor, 1976; Tezara *et al.*, 2008) as CO_2 from respiration is emitted in the light (Lawlor and Fock, 1975). Such changes in C_i/C_a have been directly measured (Lauer and Boyer, 1992), so confirming the general validity. However, as discussed, there has been concern about errors in measurements, calculations, etc, so the changes in C_i have not been accorded due weight. This has lead to contradictions in studies, e.g. Ennahli and Earl (2005), where C_i remained high and at large WD CO_2 was evolved from leaves in the light (a qualitative effect not easily explained by errors in gas exchange) so C_i must have been greater than C_a. The simplest explanation is that the initially large A_{pot} and small g_s decreased C_i, but then inhibition of A_{pot}, with maintenance of R_L, increased C_i. This is a consequence of the greater sensitivity of A than R_L to WD (see section on respiration, below). In addition, the data of Cornic *et al.* (1987) were later rejected on the basis of alternative calculations (see Cornic and Fresneau, 2002).

Chloroplast CO_2 concentration. Correct values of C_c are essential for understanding photosynthetic metabolism (von Caemmerer, 2000), and they are also required for the correct calculation of g_i. Warren (2006) has critically evaluated the several methods used for C_c, and thus g_i, calculation. All suffer from substantial and similar assumptions, so whilst providing apparently independent checks they tend to reinforce erroneous interpretations. Methods used to calculate C_c include the following.

(1) Direct measurement of exchange of C and O isotopes. This allows fluxes to be determined under WD, and from them the sinks for e^- (Haupt-Herting and Fock, 2000, 2002) and C_c (Renou *et al*, 1990; Tourneux and Peltier, 1995). Assumptions about the nature of the sinks are very important and are discussed later.

(2) Chlorophyll fluorescence. Changes in fluorescence have provided great insight into photosynthetic metabolism, including under WD (e.g. Cornic and Briantais, 1991; Cornic and Fresneau, 2002). The 'variable J' and 'constant J' methods are widely used to calculate C_c. Linear e^- transport is calculated from the quantum yield of PSII energy conversion, $\phi_{PSII} = (F_m' - F)/F_m'$), derived from maximal and steady-state fluorescence (F_m' and F, respectively), the total leaf absorptance (α) and the distribution of energy between PSII and PSI (f, usually taken as 0·5). The e^- transport is apportioned to Rubisco caboxylation and oxygenation, which, together with specificity of Rubisco, allows calculation of C_c. There are assumptions and errors in the methods, suggesting that estimation of C_c is not as quantitative as assumed. (Warren, 2006). Fluorescence is measured from chloroplasts near the tissue surface and so may not represent the population

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within the leaf, and there is strong evidence that it over-estimates e^- flux (Haupt-Herting and Fock, 2002; Tezara *et al.*, 2008). Values of α and f require measurement. Fluorescence may over-estimate the total electron transport (ET) and thus the flux to Rubisco oxygenation.

(3) A 'calibration curve' (see Lal *et al.*, 1996; Warren, 2006, 2008) of fluorescence against C_a under non-photorespiratory conditions (1–2 % O_2) is used to estimate alternative sinks (which all involve e^- transport to O_2) and to draw conclusions (Lal *et al.*, 1996) about C_c under WD. This is illogical as it assumes that fluorescence measured under WD shows C_c. There is sound, independent evidence that the relation between A and metabolism changes substantially compared to the well-watered state. As an example of the problems with the technique, and conclusions drawn from it, we hypothesize that such 'calibration' explains the absence of an effect of vapour pressure deficit, but a large effect of soil WD, on calculated C_c and g_i in three species (Warren, 2008). Clearly effects of WD differ from changes caused by [CO_2] alone, e.g. ATP decreases under WD (often assumed to cause low C_c) but increases with CO_2 deficiency (Wormuth *et al.*, 2006). Reliance on techniques that have limited independence, many basic and untested assumptions, and ignore changes in metabolism under WD is not justified.

A major assumption is in apportioning ET to photorespiration (PR), which is determined by deducting the carboxylation flux from the total, assuming alternative fluxes to be zero or very small and constant. Thus, from O_2 exchange, Renou *et al.* (1990), and Tourneux and Peltier (1995) calculated that C_c approached the compensation point, only increasing at very small WD depending on assumptions about respiration. However, judged from isotope O_2 and CO_2 exchange data (Haupt-Herting and Fock, 2002), not all ET is to PR even in turgid cells, and certainly not with WD. The small decrease in measured C_i with WD suggests that the ratio of oxygenation to caboxylation is smaller than Cornic and Briantais (1991) and Cornic and Fresneau (2002) determined. A constant flux of e^- to PR is unlikely from current knowledge of respiration and a decrease is also likely (see section on photorespiration, below). Therefore, under WD over-estimation of ET by fluorescence and under-estimation of alternative sinks probably over-estimates PR and so under-estimates C_c, the values of which must be treated with considerable caution for, as Warren (2006) says, the e^- flux is 'at best, a semi-quantitative estimate of the rate of linear electron transport'. Hence, it is legitimate to conclude that the very substantial decrease in calculated C_c with WD (Cornic, 2000; Flexas *et al.*, 2008) is an artefact, requiring rigorous examination. We suggest that with relatively small WD, C_c does decrease as C_i falls (Fig. 2D), but as WD decreases A_{pot} further, maintenance of respiration increases C_c and C_i.

Intercellular (mesophyll) conductance (g_i). Estimating the conductance, g_i, of the path (cell wall, plasmalemma and chloroplast membranes) of transport of CO_2 between the intercellular spaces (at C_i) to Rubisco in the chloroplast at C_c (Evans and von Caemmerer, 1996) is currently of major interest (Flexas *et al.*, 2008), and depends on calculation of C_c. Rapid and substantial changes in estimated g_i occur according to conditions, and are used to explain many features of CO_2 exchange under WD, for example the apparently large difference between C_c and C_i in the study by Ennahli and Earl (2005). Considering the possible role of g_i, from the model in Fig. 1, all the CO_2 released and contributing to C_i is extra-chloroplastic (no chlororespiration), so to maintain a very low C_c and large C_i would require that the large decrease in g_i would be in the chloroplast envelope, not in the plasmalemma or wall (to allow CO_2 movement to the intercellular space), requiring a specific mechanism. The uncertainty about estimating C_c must apply to g_i. Accepting that g_i reflects a real 'state' in cells, and ignoring the question as to why such a basic process should be so highly variable and sensitive to conditions, what determines g_i is still much debated. It has physical characteristics including solubility of CO_2, surface area of intercellular spaces, walls and cytosol, and dimensions of the intercellular spaces, which change as tissues and cells shrink with WD. In addition, g_i has a metabolic component (e.g. carbonic anhydrase, which facilitates CO_2 movement to Rubisco active sites: aquaporins may act as CO_2 channels), which would be changed by WD. The evidence suggests that g_i is not greatly affected by WD.

We conclude that responses of A and A_{pot} to g_s and elevated CO_2 under WD are likely to be a continuum, depending on species, growth conditions, severity and duration of WD, and the environment. At low light, metabolism is not greatly affected, nor may it be in some studies at moderate WD (e.g. Quick *et al.*, 1992) if adaptation of the stomatal response has occurred. However, with higher light, A_{pot} is generally inhibited.

METABOLIC CAUSES OF DECREASED A_{pot}

Here, we consider the effects of WD on light reactions and electron transport, including generation of reactive oxygen species and photoinhibition, followed by ATP synthesis, then Calvin cycle function, including Rubisco functions, RuBP synthesis and photorespiration. An overview of these processes is given in Lawlor (2001) and summarized in Fig. 1.

Light capture, energy use and dissipation

Light capture and energy use are central to any discussion of A and A_{pot} under WD (see Sharp and Boyer, 1985, 1986; Kirschbaum, 1987; Cornic and Briantais, 1991). When leaves are exposed to radiation, photons excite chlorophyll to the singlet excited state (^{1}chl*), which is quenched by several processes (Avenson *et al.*, 2004; Baker *et al.*, 2007), as follows. (1) Variable fluorescence from chlorophyll *a* associated with PSII. (2) Formation of triplet states of chlorophyll (^{3}chl*) by intersystem cross-over. (3) Energy-dependent quenching (q*E*), shown by non-photochemical quenching (NPQ) with energy from the antenna chlorophyll of PSII being transferred to zeaxanthin (Z) and dissipated as heat (Müller *et al.*, 2001; Kanazawa and Kramer, 2002; Niyogi *et al.*, 2005), shown as 'VAZ' in Fig. 1. Zeaxanthin accumulates when there is excess energy by conversion of violaxanthin (V) via antheraxanthin (A) catalysed by violaxanthin de-epoxidase (VDE; Fig. 1). Conversion of V to Z requires

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low pH (large $[H^+]$) in the thylakoid lumen, which activates VDE. Accumulation of H^+, and thus NPQ, is stimulated by a large proton gradient (ΔpH) across the thylakoid membrane, indicating decreased H^+ transport. This may occur as a consequence of inadequate ADP or P_i in normal metabolism, e.g. when CO_2 is limited and ATP concentration is large, but it may also occur when ATP synthase is not activated, e.g. by redox regulation of the γ-subunit of ATP synthase (Kanazawa and Kramer, 2002). Such a mechanism explains the strong negative correlation between NPQ and ATP under WD (Tezara *et al.*, 2008). (4) Photochemistry. Excitation of the reaction centres of the photosystems induces ET, with water-splitting evolving O_2 and releasing H^+ to the lumen and transport of e^- to ferredoxin and synthesis of NADPH. ET also generates the proton motive force, including ΔpH, across the thylakoid membrane required for flux of H^+ through ATP synthase, resulting in ATP synthesis (see Avenson *et al.*, 2005). NADPH and ATP are used predominantly in the Calvin cycle for CO_2 assimilation. When energy capture is in balance with photochemistry (and so there is little excess energy) fluorescence and NPQ are very small, and ^{1}chl* is rapidly quenched, minimizing the probability of generating reactive oxygen species (ROS: see Noctor *et al.*, 2002).

In unstressed leaves with rapid *A*, even quite substantial radiation flux can be used in photochemistry without causing accumulation of excess energy, and fluorescence and NPQ are very small. Complex regulation is required to ensure that these fundamental processes function under a wide range of conditions (Scheibe *et al.*, 2005; Rumeau *et al.*, 2007). However, with small WD where *A* is decreased, the same radiation may exceed the capacity of photochemistry and NPQ rises (Fig. 2I), indicating that the ET chain and redox components are over-reduced compared to the normal state (Cornic and Briantais, 1991). Increased NPQ shows that the lumen pH is very acidic and that transport of H^+ through ATP synthase is limiting. Under progressive WD this is not likely to be due to inadequate P_i (Kanazawa and Kramer, 2002; Avenson *et al.*, 2004, 2005) because ATP concentration also decreases (Tezara *et al.*, 1999) and *A* is small without accumulation of metabolites. In this respect the effects of WD differ from inadequate CO_2 supply (Wormuth *et al.*, 2006). We conclude that WD and small *A* induce over-energization of the thylakoids.

Electron transport

Energy transfer to the reaction centres of PSII and PSI results in ET to ferredoxin and then reduction of $NADP^+$. Because PSII activity is substantially maintained under WD, the potential e^- flux to acceptors is large (Cornic and Fresneau, 2002). However, as *A* progressively decreases with WD, so must consumption of NADPH. Thus, with increasing WD total ET decreases (Fig. 2H) as sink capacity falls. The reduced pyridine nucleotide content is remarkably similar with and without WD (Lawlor and Khanna-Chopra, 1984; Tezara *et al.*, 2008), not decreased as Flexas *et al.* (2004*a*) state. This is independent evidence that a crucial feature of WD is maintenance of light reactions, ET and reductant status, but with impaired ATP metabolism. We conclude that under WD, as *A* decreases substantially, ET to carboxylation falls, both absolutely and relatively to PR, decreasing these sinks for e^-. Then ET to O_2 and consumption of reductant by mitochondrial dehydrogenases (see later) become more important sinks.

Oxygen metabolism and electron transport to O_2

Electrons from the water-splitting complex enter the photosynthetic ET chain and H^+ and O_2 are released (E_o, gross O_2 evolution; the O_2 'photosynthesis' of Tourneux and Peltier, 1995). This is the sole source of O_2. However, e^- reduces O_2 via several processes, as follows. (1) Photorespiration results in the transfer of e^- to O_2 via the mitochondrial ET chain, and ATP is generated. Measurements of *A*, PR and ET and partitioning by mass spectroscopy of O and C isotopes in tomato leaves with progressive WD (Haupt-Herting and Fock, 2002) have demonstrated that E_o and gross O_2 uptake (O_u) decreased but that O_u/E_o was greater with WD, showing that O_2 reduction increased relative to O_2 evolution. Net CO_2 uptake (*A*), gross CO_2 uptake (total photosynthesis, *TPS*) and gross CO_2 evolution (all CO_2 released in the light, E_c) decreased substantially with increasing WD. Although E_c fell by approx. 40 %, E_c/TPS increased, showing greater respiratory activity, and recycling of evolved CO_2 doubled. However, *A* decreased more than E_o at severe WD so ET was maintained relative to *A*. Thus, although total ET decreases under WD it is dissociated, in part, from *A* as it reduces O_2. Tourneux and Peltier (1995) also observed, under extreme conditions, decreased E_o with increasing stress, but O_u/E_o increased from approx. 50 to 100 % with WD equivalent to 80 % RWC, below which both decreased in parallel. Such a large increase in O_2 uptake was not shown by Haupt-Herting and Fock (2002): O_u/E_o changed from approx. 50 to 60 % with WD under physiologically realistic conditions. Haupt-Herting and Fock (2002) showed that ET to O_2 increased relative to gross PR, and was not constant as expected if PR were solely responsible. Sinks for e^- include the Mehler reaction and the Asada water–water cycles, which may increase as the system becomes more reduced, but probably not greatly. A potential sink not yet explored under WD is oxidation of NADH and NADPH [transferred from the chloroplast by metabolite shuttles (Stitt, 1997) via transporters] by mitochondrial dehydrogenases (Fig. 1; see 'mitochondrial activity and water deficit', below). Quantification of sinks for e^- over a range of WD, in relation to light, is required.

Generation of reactive oxygen species (ROS)

Earlier work has been thoroughly reviewed by Smirnoff (1993), Mittler (2002) and Demmig-Adams *et al.* (2006). With WD, despite increased q*E*, components of light-harvesting, photosystems and the ET chain are produced with very negative redox potentials (Mittler, 2002; Apel and Hirt, 2004; Baier and Dietz, 2005). They react with O_2 from water-splitting (and intermediates of the process), generating ROS, including singlet oxygen ($^1O_2^*$) that results from ^{3}chl* donating energy to molecular O_2 (Krieger-Liszkay, 2005); superoxide is also formed. This reacts with H^+ in the presence of superoxide dismutase (SOD), generating hydrogen peroxide, H_2O_2, which is convert to water

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and O_2 by peroxidases. Perhydroxy radical, hydrogen peroxide and hydroxyl radical are also synthesized. ROS react with proteins and lipids, causing damage to cellular structures and metabolism, especially associated with photosynthesis. The mitochondrial ET chain and other parts of cell metabolism also produce ROS; systems for dissipation exist (Møller, 2001; Mittler, 2002) but the magnitude compared to chloroplasts under WD is unknown. Probably, generation is much greater in chloroplasts because of their larger, fluctuating energy loads. PR and the Mehler reactions generate H_2O_2 (Noctor *et al.*, 2002; Luna *et al.*, 2005), the latter using e^- from ferredoxin, a potentially important reaction during induction of *A* in allowing ET and development of the H^+ gradient for ATP synthesis (Haupt-Herting and Fock, 2002; Noctor *et al.*, 2002). Detoxification of ROS involves reactions with reduced compounds such as ascorbate and glutathione: $^1O_2^*$ is removed by reaction with tocopherol (see Asada, 2000; Mittler, 2002; and Noctor *et al.*, 2002, for detailed discussions). Detoxification mechanisms consume reducing power and form water (the 'water–water cycle', shown as 'ROS W–W' in Fig. 1; see Asada, 2000, for details). The normal capacity of the Mehler reaction to consume e^- is probably small (Biehler and Fock, 1996; Badger *et al.*, 2000; Haupt-Herting and Fock, 2002), and the water–water cycle also (Noctor *et al.*, 2002). The increase in ROS formation and concentration, and thus potential for damage, depends on the capacity of both synthesis and removal. With rapid development of WD in tissue not adjusted to the energy imbalance caused by large changes in *A*, ROS accumulation clearly depends on the balance between synthesis and dissipation, all dependent on growth conditions, rate and duration of WD, etc. However, potential damage related to ROS under WD is difficult to assess as this intricate system has not been quantified under clearly defined irradiance and WD. It is unclear if production of ROS increases substantially together with NPQ under WD, or is delayed until NPQ cannot maintain the energy status below a threshold. Blokhina *et al.* (2003) suggest that as NPQ increases so does ROS accumulation (Fig. 2I). Therefore, when WD develops over days under relatively bright light, ROS-induced damage is observed (Demmig-Adams *et al.*, 2006). Importantly, increased ROS production and the high redox state of the ET chain, etc, induces expression of genes coding for components of energy-dissipating and regulation systems in chloroplasts, allowing acclimation to conditions (Pfannschmidt *et al.*, 2009).

Photosystem activity and photoinhibition of PSII and ATP synthase

Effects of WD were analysed by Sharp and Boyer (1986), Kirschbaum (1987) and Demmig-Adams *et al.* (2006). Over a wide range of WD, excitation of the antenna chlorophylls and PS reaction centres is maintained, although some decrease in energy transfer, shown by smaller F_o during illumination, may occur in the antenna (Haupt-Hertung and Fock, 2002). Efficiency of PSII measured in dark-adapted leaves by F_v/F_m is generally unimpaired by WD (Fig. 2J; see Tezara *et al.*, 1999) unless severe. Photochemical quenching (q*P*), a measure of efficiency of PSII, decreases at 60 % RWC, when *A* is very small. Thus PSII is not impaired by relatively severe WD: this also applies to PSI. Cornic and Briantais (1991) concluded that PSII activity is much less affected by WD than other partial processes in photosynthesis, justifying their view that 'photosynthesis' is not sensitive to WD. Insensitivity of PSII to WD is surprising as it is susceptible to damage (photoinhibition, PI), by $^1O_2^*$ attack on its *D*1 (32 kDa) protein, which is very labile and rapidly turned-over (half-life approx. 2 h). The propensity to PI, seen at low CO_2 and O_2 in bright light, is regarded as a feature of WD, on the assumption (e.g. Kanazawa and Kramer, 2002) that C_c is close to the compensation point. However, evidence for PI during WD is equivocal. Sharp and Boyer (1986) demonstrated in sunflower that quantum yields of CO_2 fixation and rates of light- and CO_2-saturated *A* decreased substantially with WD, but were not affected by the light intensity during increasing WD, so that PI did not occur. However, PI developed when CO_2 and O_2 were almost absent. Kirschbaum (1987) observed that WD decreased the A/C_i relationship in *Eucalyptus pauciflora* but PI was not a major contributor to it. In contrast, Lu and Zhang (1998) concluded that when WD was imposed gradually on wheat at low light, *A* and g_s decreased significantly without affecting PSII photochemistry or maximal efficiency, and without damaging reaction centres or antennae. However, photochemistry was affected after light adaptation, with decreased efficiency of excitation-energy capture by open PSII reaction centres and quantum yield of PSII ET, and without a significant increase in NPQ and increased PI. Giardi *et al.* (1996) observed damage to the *D*1 protein, indicated by decreased q*P* and F_v/F_m, under WD, with excessive energy load.

Damaged *D*1 protein is rapidly degraded and new replacement is synthesized and incorporated into PSII reaction centres by mechanisms involving chaperones. Re-synthesis is an important rate-limiting step (see Nishiyama *et al.*, 2001; Yokthongwattana and Melis, 2006; Takahashi *et al.*, 2007; Saibo *et al.*, 2009) but has not been examined under WD. The gene *PsbA* is chloroplast encoded, suggesting that regeneration might be very susceptible to conditions in the chloroplast, particularly if ATP is limiting under WD. Perhaps damage to PSII is limited by NPQ, or locally by cyclic ET (Rumeau *et al.*, 2007) and by the relatively protective lipid membrane. Regulation of chloroplast (and PSII) energetics is complex (Avenson *et al.*, 2005; Rumeau *et al.*, 2007), with cyclic ET around PSI and modulation of H^+ efflux through ATP synthase by 'sensing' of stromal metabolites. As metabolites change drastically with WD, the potential for unbalanced regulation is large (Joët *et al.*, 2002). Differences between experiments might be related to differences in the radiation load, susceptibility to damage and rate of repair significantly interacting with WD.

Photoinhibitory damage to ATP synthase is a recently described phenomenon, of great potential importance under WD. The γ-subunit of the ATP synthase complex was preferentially attacked by $^1O_2^*$ in a conditional mutant *flu* of *Arabidopsis* (Mahler *et al.*, 2007), which accumulated protochlorophyllide in darkness and so generated $^1O_2^*$ upon illumination. Damage was close to two regulatory cysteine molecules C178 and C184: the γ-subunit is not surrounded by other proteins and is thus potentially exposed to attack. Damage correlated strongly with a decrease in ATP hydrolysis activity and with increased NPQ (Mahler *et al.*, 2007). As ATP

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hydrolysis correlates strongly with ATP synthase activity, it suggests that loss of ATP synthase activity may occur under high light and WD when ROS is generated. Direct evidence that WD damages chloroplastic ATP synthase, with the ε-subunit lost from thylakoids to the stroma, is provided by Kohzuma *et al.* (2008). Normally the ε-subunit binds to the γ-subunit and suppresses ATPase activity, and may have a role in relaxation of the hyper-energized state and regulation of proton movement through the complex. With over-energized conditions, as with WD, loss of the ε-subunit may allow relaxation of hyper-energization and dissipation of the ΔpH and proton motive force, changing energy coupling (Akashi *et al.*, 2004).

Different species of ROS affect other subunits of ATP synthase, e.g. H_2O_2 impairs the α- and β-subunits, but more slowly than $^1O_2$ damages the γ-subunit.

However, PI damage to ATP synthase remains to be demonstrated under WD. We hypothesize that ATP synthase is damaged and then removed from thylakoids, resulting in the decreased content observed by Tezara *et al.* (1999). Possibly, the repair cycle for ATP synthase components is not as active as for *D*1 protein (Nishiyama *et al.*, 2001) or inhibition decreases ATP synthesis, so slowing and disrupting the re-synthesis and repair cycles. PI-related inhibition, resulting in damage to ATP synthase with relatively mild WD observed in isolated chloroplasts (e.g. Keck and Boyer, 1974) and in intact leaves where ATP synthase protein is lost (Tezara *et al.*, 1999), would explain decreased ATP content and A_{pot}, accounting for the differences associated with dim and bright light during WD (Table 1). This testable hypothesis requires that ATP synthase is more sensitive to PI than is PSII, because loss of ATP synthase was detected at WD where F_v/F_m was unaffected, and q*P* was still large (Tezara *et al.*, 1999). ATP synthase may be more sensitive than PSII to attack by ROS, either because of differences in molecular structure, or it is more accessible to ROS then *D*1 protein. In contrast with the lipid environment of PSII, the progressive increase in ionic concentrations, particularly of Mg^{2+} during WD (Younis *et al.*, 1979, 1983), in the aqueous environment of the ATP synthase complex may enhance damage. Interestingly, this may relate to the question of why chloroplast genes have migrated to the nucleus and the role of stress conditions in the process (Cullis *et al.*, 2009). There is a *prima facie* case that ATP synthase is inhibited by conditions that occur under WD. A detailed, objective examination of the problem is needed.

ATP metabolism under water deficit

We consider that ATP synthesis is of crucial importance to understanding effects of WD. Early evidence of impaired ATP synthase in isolated chloroplasts (see Keck and Boyer, 1974; Tang *et al.*, 2002) was largely ignored or dismissed, but substantiated by evidence from intact leaves (Barlow *et al.*, 1976; Lawlor and Khanna-Chopra, 1984; Tezara *et al.*, 1999, 2008), including for marama bean under WD (M. Searson and M. J. Paul, pers. comm.; see Mitchell *et al.*, 2005). That WD has different effects from changing C_i is shown by the data of Wormuth *et al.* (2006) in a study of gene expression and metabolic regulation in *Arabidopsis* subjected to different C_a over several hours, where concentrations of ATP and ATP/ADP ratio were large with zero CO_2 but decreased in elevated CO_2. ATP accumulation is to be expected where the main sink for e^- (CO_2) is removed yet ET and H^+ transport are unaffected. Decreased A_{pot} (Fig 2G) correlated strongly with decreased ATP (Fig. 2E; Tezara *et al.*, 1999). The evidence and explanation (Lawlor, 1995: Tezara *et al.*, 1999; Lawlor 2002), that ATP content ultimately limits A_{pot}, is not accepted by Flexas *et al.* (2004*a*, 2006), mainly on the grounds that ATP has not been sufficiently measured (suggesting the need for rectification using proper experimentation and sampling), or decreases only at very large WD and so has no importance for *A*. They also believe that PR increases, thus increasing ATP, although ATP from dark respiration decreases due to inhibition of mitochondrial ATP synthase, rather than that of chloroplasts.

An indirect measure of ATP synthesis failed to demonstrate any effect of WD in a study by Ortiz-Lopes *et al.* (1991) on sunflower growing in the field. They demonstrated activation of ATP synthase at severe WD from measurement of the decay in the flash-induced electrochromic absorption change at 518 nm from cytochrome, caused by H^+ flux from the inner thylakoid space to the stroma through the activated ATP synthase (CF_o–CF_1). However, it is not clear that change in the signal is quantitatively related to ATP synthesis. Loss or malfunction of some ATP synthase complexes, with activation of those remaining, albeit with altered decay kinetics (which were observed), could decrease ATP synthesis. No confirmatory measurements of ATP content were made. These interpretations cannot be reconciled with the evidence of a decrease in ATP measured under WD. Clearly, as the main sinks (CO_2 assimilation, protein synthesis) for ATP are strongly decreased under WD, yet ATP content decreases, inhibition of ATP synthesis occurs, rather than increased consumption.

Role of ATP synthase in metabolic regulation

Increasingly, ATP synthase activity is regarded as regulating *A*, ET, energy, NADPH and ATP balance under greatly and rapidly changing environmental conditions (Herbert, 2002), and we suggest particularly so under WD. Mechanisms are discussed by Dal Bosco *et al.* (2004), Avenson *et al.* (2005), Wu *et al.* (2007) and Takizawa *et al.* (2008). Using spectroscopic techniques, Kanazawa and Kramer (2002) demonstrated that the H^+ flux through ATP synthase is slowed by factors (possibly stromal metabolites or P_i) other than the redox regulation of the γ-subunit when C_a is altered and *A* of leaves decreases. Lumen pH is responsible for the large NPQ so a strong inverse correlation between NPQ and ATP, as observed by Tezara *et al.* (2008), is expected if impaired ATP synthesis slowed H^+ transport. Dal Bosco *et al.* (2004) showed that inactivation of the ATP synthase γ-subunit prevented ATP synthesis under reducing conditions in *Arabidopsis* and increased NPQ substantially due to a large H^+ gradient. This is further evidence that damage to ATP synthase would produce responses similar to WD.

Calvin cycle under WD

Function of the Calvin cycle is central to CO_2 assimilation (von Caemmerer, 2000; Lawlor, 2001). Carbon flux through

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this complex system depends on many processes and is highly regulated. Analysis has been limited, with focus on amounts of RuBP and 3PGA, and on Rubisco. Measurements have demonstrated that RuBP decreases with WD (Giménez *et al.*, 1992; Gunasekera and Berkowitz, 1992; Tezara *et al.*, 1999), and 3PGA also (Lawlor and Fock, 1977; Tezara *et al*, 1999). However, Flexas *et al.* (2004*a*, 2006) did not consider that WD affected the RuBP content. Wingler *et al.* (1999) measured approx. 25–30 % decrease in A and 50–60 % in RuBP and 3PGA, although they considered RuBP not to be limiting as it was above estimated concentrations of Rubisco binding sites. With WD, if A_{pot} is not affected, decreased A as a consequence of low g_i should increase RuBP and, particularly, ATP contents as they are not consumed, provided that amounts and activities of Calvin cycle enzymes are maintained (Fig. 1). If the enzymes of the cycle are impaired then RuBP should decrease and ATP rise; however, the decrease in RuBP and ATP suggests that the supply of ATP is inadequate. Regulation was discussed by Tezara *et al.* (1999) and Lawlor (2002), who concluded that ATP supply was the limiting factor.

Rubisco amount and activity

Rubisco amount and, particularly, activity under WD have been extensively studied (see Parry *et al.*, 2002; Flexas *et al.*, 2006). Changes in Rubisco amount and activity are variable between studies and not well correlated with changes in A or metabolites. Generally, Rubisco protein content per unit area of young, mature leaves does not decrease until WD is severe (Fig. 2E; e.g. Giménez *et al.*, 1992; Wingler *et al.*, 1999; Tezara *et al.*, 1999); indeed, it may increase due to leaf shrinkage, although it does decrease in some studies, for reasons unknown (Tezara *et al.*, 2002). Rubisco activity (initial) does decrease with severe WD in some studies (see Flexas *et al.*, 2004*a*, 2006), suggesting that it may limit A. Wingler *et al.* (1999) found no decrease in Rubisco activity or activation state despite a large decrease in A with WD in barley. Bota *et al.* (2004) concluded that Rubisco was more affected by WD than RuBP synthesis, but conditions, sampling, etc., were probably inadequate. There were no data on ATP. Flexas *et al.* (2006) measured initial activity of Rubisco in *Glycine max* and *Nicotiana tabacum* and correlated it with calculated C_c, substantiating their view that low C_c inhibits Rubisco. Judged from the very similar changes in RuBP and 3PGA (Fig. 2G), and RuBP and ATP (Fig. 2E, G; e.g. Tezara *et al.*, 1999), but not in Rubisco, the enzyme does not alter the flux in the Calvin cycle under WD, but other conditions are probably also important (e.g. nitrogen supply).

Analysing regulation of Rubisco is difficult because both amount (determined by breakdown and synthesis) and enzymatic activity change, the latter particularly rapidly. Inhibitors such as analogues of RuBP bind to its active sites, especially at sub-saturating RuBP concentration under WD (Giménez *et al.*, 1992; von Caemmerer, 2000). They are not easily displaced, so that Rubisco activity diminishes. It is restored and regulated by Rubisco activase, a catalytic molecular chaperone that removes inhibitors from the active sites, and requires a large ATP/ADP ratio: as this drops Rubisco activase and Rubisco activity decrease, so slowing Rubisco (Parry *et al.*, 2002; Portis, 2003). Activase is also regulated by redox changes mediated by thioredoxin-f, which alters the response to the ATP/ADP ratio (Portis, 2003). To summarize: loss of Rubisco activity in WD seems more likely to be related to Rubisco activase and lack of ATP than to changes in protein, although the latter is possible.

Sucrose synthesis

Sucrose, starch and 3PGA contents usually fall (Fig. 2F) with progressive WD and decreasing A in sunflower (e.g. Lawlor and Fock, 1977). In *Phaseolus* (Vessey and Sharkey, 1989) a moderate WD (−1 MPa) decreased A and sucrose synthesis by 70 %, starch by 12-fold, and substantially decreased the A/C_i response, removing O_2 sensitivity of A. Sucrose phosphate synthase (SPS) activity fell by 60%. Low C_a also decreased SPS in well-watered tissue; this was reversible by large C_a but not under WD. However, Quick *et al.* (1989) observed stimulation of SPS activity with WD at large C_a. The contradiction was explained by SPS responding to A, with g_s the main control. Vessey *et al.* (1991) concluded that CO_2 supply'... explains away the last support ... for direct effects of water stress on photosynthesis ... 'but this is not justified. Flexas *et al.* (2004*a*) found the reasons for the greater inhibition of starch than sucrose synthesis with WD 'not clear'. We suggest that the most important factor is the substantial fall in A, limiting synthesis of substrates, followed by changed regulation, consistent with the known properties of enzymes. Starch synthesis falls precipitately as [3PGA] (Lawlor and Fock, 1977) and ATP decrease and P_i rises (likely but not proven) due to inhibition of ADP glucose pyrophosphorylase activity. Sucrose synthesis decreases because the small concentration of glucose-6-phosphate (G6P) activates SPS kinase, and increased P_i inactivates SPS phosphatase: both effects inactivate SPS (Huber and Huber, 1996). Large C_a at such small WD would probably increase G6P, and thus SPS activity. As WD increases, so inadequate ATP and G6P and increased P_i become more dominant, in addition to low A. The system will be very dependent on conditions. Experimental evidence about metabolites and enzyme activity is consistent with known regulatory mechanisms, and shows that enzyme activities follow metabolism under WD, not regulate it. The conclusion of Flexas *et al.* (2004*a*) that lack of response to elevated CO_2 is only explicable by 'a functional limitation ... [of] starch-sucrose synthesis' is not justified and is supported by flawed modelling, with many erroneous assumptions.

Photorespiration (PR)

As A is decreased by falling g_s, PR (which is approx. 25 % of A under normal conditions) becomes relatively more important under WD (Fig. 2C; Lawlor and Fock, 1975; Lawlor, 1976; Wingler *et al.*, 1999; Haupt-Herting and Fock, 2002; Noctor *et al.*, 2002) or may entirely replace the decreased A (Cornic and Fresneau, 2002). PR (and PR/A) rise as a consequence of the Rubisco oxygenase reaction, which is determined by the ratio of $[O_2]/[CO_2]$ at the catalytic sites of Rubisco (see Fig. 1). Phosphoglycollate from the oxygenase reaction is metabolized to glycine, which is

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decarboxylated by glycine decarboxylase in the mitochondria, producing serine, with release of CO_2 (PR) and e^-: the latter are transferred to O_2 via the mitochondrial ET chain thus generating ATP (Fig. 1). As mentioned earlier, the view of Flexas *et al.* (2004*a*) and Ribo-Carbo *et al.* (2005) is that ATP production by mitochondria decreases with WD. Experimental evidence suggests that PR is not as large an absolute sink for e^- as once thought, but the assumption (or dogma) that it increases substantially is largely unquestioned (e.g. Kanazawa and Kramer, 2002). PR either remained rather constant over a wide range of WD or decreased when measured on rapidly stressed sunflower leaves by means of CO_2 exchange with 21 and 1 % O_2 – allowing and preventing PR, respectively, although PR/*A* increased (Lawlor, 1976; Lawlor and Fock, 1975). Using $^{12}CO_2$ and $^{13}CO_2$ exchange, Haupt-Herting and Fock (2000, 2002) showed that PR changed little, although PR/*A* increased. Given the very large decrease in *A*, the total sink ($A + PR$) for e^- is much decreased. Earlier measurements of respiration in the light by CO_2 exchange did not adequately account for light respiration, which increased as a proportion of total respiratory CO_2 emission determined by $^{14}CO_2$ measurements and the decrease in specific radioactivity of CO_2 emitted (Lawlor and Fock, 1975) and this overestimated PR under WD. This shows that stored C reserves were consumed with WD, in agreement with a substantial fall in sucrose content (Lawlor and Fock, 1977). In addition, the increase in equilibrium CO_2 compensation concentration with WD (Γ, Fig. 2D) was unaffected by 1 % O_2, i.e. the CO_2 was not from PR (Lawlor, 1976). The evidence of Wingler *et al.* (1999), based on enzyme and metabolite assays, that PR increased in barley under WD is not consistent with smaller glycine and serine contents and a constant gly/ser ratio: PR CO_2 exchange was not measured. As RuBP synthesis decreases with WD so both oxygenase and carboxylase reactions will decrease, although the O_2/CO_2 ratio affects PR/*A*. We conclude that the total sink for e^- provided by PR does not increase with progressive WD, so its role (whilst becoming relatively more important than *A*) in energy regulation is over-estimated. Respiration from the carboxylic acid cycle in the mitochondria becomes relatively more important.

Mitochondrial activity and water deficit

The vital role of mitochondria in photosynthetic carbon metabolism of leaves experiencing WD is considered by Atkin and Macherel (2009), so it is only briefly emphasized here. Mitochondria are responsible for respiration [tricarboxylic acid (TCA) cycle] in darkness – dark respiration, R_D – and in the light (R_L) and PR, and e^- from these processes reduces O_2, forming water (Fig. 1) and transporting H^+, which ultimately results in ATP synthesis (Vedel *et al.*, 1999). Another essential feature is that mitochondria use NADH and NADPH (including from the chloroplast), transferring e^-, and are thus particularly important in redox regulation (Rasmusson and Møller, 1990; Lin *et al.*, 2008). The multiplicity of dehydrogenases in plant mitochondria and how they function in redox regulation is discussed by Rasmusson *et al.* (2008). The redox state ($NADH + NADPH/NAD^+ + NADP^+$) under WD is comparable to normal conditions (Lawlor and Khanna-Chopra, 1984; Tezara *et al.*, 2008), yet *A* is inhibited, suggesting over-reduction. We suggest that NAD(P)H is dissipated by mitochondria even at rather mild stress and is probably a major sink for electrons originating in the light reactions, with shuttle systems transferring reductant across the chloroplast envelope to the mitochondria (Stitt, 1997), although there is lack of evidence under WD. Synthesis of ATP in mitochondria is probably very important for ion transport and protein synthesis in the cytosol and it may be available to chloroplasts via efficient membrane transporters (Stitt, 1997).

If the flux of reductant from the chloroplast exceeds the capacity of the mitochondria to generate ATP, reductant may still be dissipated by the mitochondrial alternative oxidase (AOX), which uses e^- to reduce O_2 but without coupling to ATP synthesis. AOX activity depends on a highly reduced state (Vedel *et al.*, 1999) so it becomes more important under WD. There is clear evidence of this in wheat leaves, as the amount of reduced and active AOX protein increased substantially (Bartoli *et al.*, 2005). Inhibition of AOX did not affect fluorescence in well-illuminated and well-watered leaves, but with WD, ϕPSII and q*P* decreased and NPQ increased greatly, especially when AOX was inhibited, although F_v/F_m was unaffected (Bartoli *et al.*, 2005), indicating that AOX maintains photosynthesis under WD. More active AOX, by dissipation of e^-, decreases ROS production, whilst decreasing oxidative phosphorylation (Ribo-Carbo *et al.*, 2005). This is taken as evidence by Flexas *et al.* (2006) that WD inhibits ATP synthesis by mitochondria. It is more likely that ATP synthesis is maximal from mitochondria and that the excess e^- is used by the AOX when ubiquinone is over-reduced. Further control of the redox and ATP status of the cell and organelles is provided by mitochondrial uncoupler proteins (UCP), which allow H^+ to flow without passing through ATP synthase, so preventing ATP synthesis if ATP consumption is inadequate (Sweetlove *et al.*, 2006), but this is not the case under WD. However, UCP is also activated by superoxide (ROS), and is required for the oxidation of glycine to serine in the photorespiratory pathway. Thus, conditions in the cell under WD might activate AOX and UCP as part of regulation (Clifton *et al.*, 2006). We conclude that mitochondria provide several 'safety valves' allowing balance to be achieved between e^- and H^+ fluxes, PR and TCA respiratory pathways, and between NAD(P)H and ATP synthesis. It is a matter of urgency to properly evaluate the magnitude of the different sinks for e^-.

Chloroplasts are much more sensitive to WD than mitochondria because when *A* stops R_L is maintained (Lawlor and Fock, 1975), although ROS is formed in both organelles, and Bartoli *et al.* (2004) consider mitochondria to be sensitive to ROS. Regulatory systems for dealing with excess e^- are different in the two organelles: multiple regulatory pathways in mitochondria may provide greater protection. Perhaps the range of energy states is smaller in mitochondria than in chloroplasts, which experience large and rapidly changing radiant energy fluxes. Chloroplasts (particularly those not adjusted to strong light, WD, etc) may have inadequate mechanisms to prevent the accumulation of H^+ and to dissipate the H^+ gradient if it is large, except through ATP synthase. ATP synthases from both organelles, which have a different evolutionary origins

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and structure in plants (Hamasur and Glaser, 1992), may also differ in susceptibility to their environment, e.g. ROS and ion concentrations. How conditions in the organelles affect use of reductant and generation of ATP is not known: these topics deserve more attention in the context of WD.

SUMMARY OF REGULATION OF PHOTOSYNTHETIC METABOLISM UNDER DROUGHT STRESS

This analysis of the literature shows that the relative effects of stomatal and metabolic limitations (g_s and A_{pot}) depend on species and conditions of growth and experimentation. It has led to development of a qualitative 'conceptual model' that accommodates many experimental data and generates specific hypotheses. Under WD the balance between energy capture and metabolism is disturbed, as photochemistry decreases and energy dissipation increases. With mild, relatively rapid WD and decreased g_s, A falls substantially and C_i and C_c a little. The magnitude of the change in C_c may be exaggerated because of assumptions and errors in measurements, so g_i is not reliable. Light reactions, ET and $NADP^+$ reduction are maintained, causing energy imbalance under mild stress. We hypothesize that this results in synthesis of ROS, which damages ATP synthase due to an interaction with increased ion concentrations in the chloroplast. Damaged complexes are removed from thylakoids while undamaged complexes continue to transport H^+ and synthesize ATP. The ensuing decrease in ATP is crucial to chloroplast functions, slowing RuBP synthesis, which then results in loss of metabolic potential (A_{pot}) with an accompanying decrease in Rubisco activity. Changes in Rubisco are not sufficiently well correlated with A to suggest that it has a primary role under WD. The model accounts for decreased A, A_{pot} and increased NPQ, as well as loss of ATP synthase and low ATP content. It also explains changes in metabolites. Stimulation of A by elevated C_a occurs in weak light, where damage to ATP synthase would be minimal, but does not occur in strong light, where ATP synthase is damaged. As A drops, R_L is maintained (or may increase), eventually exceeding A: consequently C_i/C_a increases greatly. As a consequence of slowed A combined with decreased ATP, metabolites of the Calvin cycle decrease, and sucrose also. There is a large increase in energy dissipation (NPQ), and maintenance of reductant content and a substantial decrease in ATP/reductant. A crucial test of the model would be to increase ROS production (e.g. by using the conditional *flu* mutant; Mahler *et al.*, 2007) or to decrease energy dissipation using a mutant, and subject plants to controlled WD under a range of defined radiation. Increased ROS or decreased dissipation would greatly increase sensitivity to WD at low light, and particularly so at high light. The effect would be seen in increased ROS, which would precede the decrease in ATP synthase amount and activity, and decreased ATP content. As a result, A/C_i responses and metabolites would be altered as described. Concomitant measurement of Rubisco amount and activity would test if it changes in relation to A and A_{pot}. We have focused on C_3 plants, but the metabolic responses and sensitivity of C_4 plants to WD, reviewed by Ghannoum (2009), suggest that the C_3 cycle in C_4 metabolism is impaired. We hypothesize that damage to ATP synthesis is the determining process in C_4 photosynthesis: this is similarly testable. Explanation of the effects of WD and tests thereof requires the correct measurement of, amongst other things, A, A_{pot} and NPQ, and analysis of metabolites, particularly RuBP, ATP, ADP and P_i, using rapid freeze-clamping, extraction, etc, under strictly comparable conditions in order to allow objective comparison of data. This model is very dynamic, considers environmental factors, and explains many observations in the literature without precluding any: it introduces flexibility into current interpretation.

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